THIS IS NOT DR. CONN'S ALDOSTERONE ANYMORE

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ABSTRACT

In 1955, Dr. Jerome Conn described a patient with severe hypertension and hypokalemia and an aldosterone-secreting adenoma. The prevalence of hyperaldosteronism is increased among patients with obesity or resistant hypertension. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers reduce the secretion of aldosterone, but with chronic treatment aldosterone concentrations "escape" back to baseline values. Mineralocorticoid receptor (MR) antagonism reduces mortality in patients with heart disease who are already taking an ACE inhibitor and diuretic. In addition to affecting sodium and potassium homeostasis via classical MR-dependent pathways, aldosterone induces inflammation and causes cardiovascular remodeling and renal injury. Some of these effects involve MR-independent pathways. At the same time, ligands other than aldosterone can activate the MR. This paper reviews mechanism(s) for the proinflammatory and profibrotic effects of aldosterone and presents data indicating that endogenous aldosterone, acting at the MR, contributes to many of the pro-inflammatory and pro-fibrotic effects of angiotensin II *in vivo.*

In 1955, Jerome Conn, University of Michigan, described the case of a 34-year-old woman with severe hypertension and a low serum potassium level at the American Society for Clinical Investigation meeting in Atlantic City (1). The patient had excessive aldosterone in her urine, as it was measured at that time in equivalents of deoxycortone acetate per day. The patient had an adrenal adenoma. In recent years, the prevalence of hyperaldosteronism has been reported to be increased (2). This is not due to an increase in the incidence of adenomas, but is perhaps related to inappropriately high aldosterone concentrations in obesity (3). Indeed, Dr. Ted Goodfriend of the University of Wisconson and others have described increased aldosterone concentra-

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tions in obese patients and a correlation between aldosterone concentrations and markers of insulin resistance (4, 5). Aldosterone may be produced at extra-adrenal sites including the vasculature (6), and products of adipose tissue increase aldosterone synthesis (7, 8). At the same time, hyperaldosteronism has been associated with sleep apnea and resistant hypertension (9–11). More importantly, mineralocorticoid receptor (MR) antagonists have proven extremely effective in reducing blood pressure in patients with resistant hypertension (9, 12).

Classically, aldosterone acts at epithelial cells such as the principal cell via the MR, resulting in translocation of the ligand-activated receptor into the cell nucleus and activation of the transcription of genes such as those for serum-and-glucocorticoid kinase (SGK), which ultimately results in activation of the epithelial sodium channel (ENaC) and the retention of sodium and excretion of potassium (13). We now understand that aldosterone also exerts effects at non-epithelial cells, and contributes to cardiac fibrosis, perivascular inflammation, and glomerular injury (14). The seminal work of Karl Weber showed unequivocally that aldosterone induces cardiac fibrosis in the setting of high salt concentrations, and that MR antagonism is protective against this (15). Later, Ricardo Rocha and others demonstrated that this fibrosis was preceded by an acute inflammatory response, characterized by the infiltration of macrophages and CD3+ T-cells into the heart and kidney, for example (16, 17).

Work in our laboratory has focused on the mechanism through which aldosterone-induced inflammation progresses to fibrosis. We and others have shown that aldosterone increases the expression of plasminogen activator inhibitor-1 (PAI-1) in a variety of cell types (18–21). PAI-1 inhibits tissue plasminogen activator (tPA) and the formation of plasmin (22). Plasmin activates the metalloproteinases (23), but also activates latent transforming growth factor (TGF)-β (24). By decreasing the formation of plasmin, PAI-1 can either promote or prevent fibrosis (25–28). Independent of its inhibitory properties, PAI-1 can affect cell migration by binding to vitronectin (29).

Through a series of experiments in PAI-1-deficient mice or in mice treated with a pharmacologic PAI-1 inhibitor, we have found that PAI-1 contributes to aldosterone-induced glomerular injury as well as to angiotensin II (Ang II)-induced vascular remodeling (30, 31). In contrast, we and many other groups have observed that PAI-1 deficiency or inhibition increases interstitial fibrosis in the heart, suggesting that PAI-1 exerts an anti-fibrotic effect in the heart (30–34). Recent data from Dr. Doug Vaughan's group at Northwestern fur-

ther suggests that this relates to unbridled activation of TGF-β in the heart (35).

A persistent question in this area is whether aldosterone *per se* or MR activation contributes to cardiovascular and renal injury. On the one hand, aldosterone has been reported to exert MR-independent effects in the vasculature (36). On the other, MR antagonism decreases inflammation and fibrosis even during high salt intake, when endogenous aldosterone concentrations are relatively suppressed (37), and ligands other than aldosterone may activate the MR. For example, Ang II can activate the MR directly, through an angiotensin I (AT_1) receptor-independent effect (38). In addition, cortisol may occupy the MR in non-epithelial cells, because many of these cells, such as cardiomyocytes, lack the enzyme 11β-hydroxysteroid dehydrogenase (HSD) 2, which converts the MR ligand cortisol into the non-ligand cortisone (39, 40).

Much of the controversy around the relative importance of aldosterone versus MR activation has evolved from studies of cardiac fibrosis and the role of the cardiomyocyte MR. Paradoxically, systemic administration of MR antagonists decreases cardiac fibrosis (37), whereas cardiomyocyte-specific MR deficiency does not, and cardiomyocyte 11β-HSD2 overexpression actually worsens cardiac injury (39, 41). This controversy may have resulted from a lack of appreciation of the central role of the macrophage in cardiac fibrosis. Usher et al have demonstrated that macrophage-specific knockout of the MR prevents cardiac hypertrophy and fibrosis in response to combined treatment with Ang II and a nitric oxide synthase inhibitor (42).

Understanding the relative contribution of aldosterone versus MR activation in the pathogenesis of cardiovascular remodeling and renal injury now assumes some clinical relevance, as aldosterone synthase inhibitors are under development. One of these, FAD286, was originally developed as an aromatase inhibitor before it was discovered to inhibit aldosterone synthase (43). Fiebeler and colleagues have shown that FAD286 decreases cardiac hypertrophy in rats (44). Recently, another aldosterone synthase inhibitor has been shown to decrease aldosterone concentrations in patients with primary hyperaldosteronism (45). In this paper, I describe a series of studies in which we used the aldosterone inhibitor FAD286, the MR antagonist spironolactone, and aldosterone synthase deficient (AS$-/-$) mice (46) to test the hypothesis that endogenous aldosterone contributes to end-organ damage.

MATERIALS AND METHODS

Comparative effect of MR antagonism and aldosterone synthase inhibition on end-organ damage. Eight-week-old male Sprague-Dawley rats were randomized to one of seven treatment groups: (1) sham-operated control rats provided with 1% NaCl in their drinking water with vehicle given via an osmotic mini-pump (Alzet; Alza Inc., Palo Alto, CA) and placebo chow; (2) uninephrectomized control rats given 1% NaCl, vehicle, and placebo chow; (3) uninephrectomized rats given 1% NaCl, vehicle, and spironolactone in their chow; (4) uninephrectomized control rats given 1% NaCl, vehicle, and FAD286 in their chow; (5) uninephrectomized rats given 1% NaCl, Ang II (1 μg/hr) by mini-pump, and vehicle in their chow; (6) uninephrectomized rats given 1% NaCl, Ang II, and spironolactone in their chow; and (7) uninephrectomized rats given 1% NaCl, Ang II, and FAD286 in their chow (47). The drugs used in the study were formulated in standard rodent chow by TestDiet (Richmond, IN) at concentrations calculated to provide doses of 5.8 mg/kg/d spironolactone and 4 mg/kg/d FAD286 as the latter's HCl salt.

Uninephrectomy and mini-pump implantation were performed at randomization and pumps were reimplanted at 4 weeks. Blood pressure was measured before randomization and every 2 weeks thereafter for 8 weeks, using tail-cuff plethysmography (BP-2000 Blood Pressure Analysis System; Visitech Systems, Apex, NC) in unanesthetized, trained rats prewarmed for 30 minutes at 37°C. Carotid-artery catheters were inserted for direct arterial blood pressure measurements at the time of pump implantation in a few animals within each group. Blood was collected via the saphenous vein under isoflurane anesthesia before randomization and at 4 and 8 weeks afterward. Rats were housed in individual metabolic cages (Nalgene®; Braintree Scientific, Braintree, MA) at 0, 4, and 8 weeks for 24-hour urine collection.

Animals were euthanized with pentobarbital, and blood was collected via cardiac puncture. The renal artery was clamped and blood was drawn from the right ventricle. The base of the heart, the descending aorta, the adrenal glands, and sections of kidney and liver were fixed overnight in 4% buffered paraformaldehyde and were then processed and embedded in paraffin for histologic evaluation. The remaining heart, aorta, kidney and liver were snap-frozen in liquid nitrogen for mRNA analysis. Further details of the study specimen collection have been previously reported (47).

Contribution of endogenous aldosterone to inflammatory gene expression following acute Ang II infusion. Eight- to 14-week-old male aldosterone synthase-deficient (AS−/−) mice and wild-type (WT) littermates were housed in metabolic cages while ingesting normal chow and water ad libitum for 24-hour urine collection (48). One week later (3–5 days before the infusion study), mice underwent placement of catheters in the right jugular vein and left carotid artery. Catheter patency was maintained by daily flushing with 50 μL of 20% heparinized saline. On the morning of the infusion study, ambulatory blood pressure was measured via the arterial catheter, with a tether-and-swivel system (BPA Analyzer; Micro-Med, Inc., Louisville, KY). To permit blood pressure stabilization, baseline blood pressure was taken 30 minutes after connection of the transducer system. Mice were randomized to receive either Ang II (600 ng/kg/min; CalBiochem, La Jolla, CA) or 0.9% sodium chloride solution at a similar rate (1 μL/25 g/min; Harvard Apparatus, Holliston, MA) for 4 hours.

To ensure that any differences observed in AS−/− as compared with WT mice resulted from a lack of aldosterone rather than from an artifact of gene manipulation, we conducted a "reconstitution experiment" in which we added back basal concentrations of aldosterone. Mini-osmotic pumps (Alzet Model 2001; Alza Inc.) containing either aldosterone (0.021 mg/mL in 2% methyl sulfoxide; Acros Organics, NJ) or vehicle were implanted subcutaneously at 7 days before the infusion study in AS−/− mice (AS−/−-aldosterone 7-day) anesthetized with pentobarbital at 50 mg/kg. The aldosterone solution and vehicle were each infused at a rate of 500 ng/day. In additional sets of experiments, we also administered aldosterone intravenously (250 ng over 4 hours) during the 4-hour infusions of Ang II in AS−/− mice (AS−/−-aldosterone 4-hour), to mimic the acute increase in aldosterone that occurs in WT mice during Ang II infusion.

Following infusion, mice were sedated with pentobarbital at 50 mg/kg IV. The left renal artery was clamped, and blood was collected passively into dipotassium-EDTA (Microvette CB K2E; Sarstedt AG & Co., Numbrecht, Germany) and centrifuged at 6,000 rpm for 5 minutes, and the plasma was stored immediately at −80°C. The base of the heart, the first 2 mm of descending aorta, and coronal sections of the kidney were fixed overnight in 4% buffered paraformaldehyde and embedded in paraffin. An additional sample of each tissue was placed in a 30% sucrose gradient for ~1 hour before embedding in paraffin for autoradiography. The remainder of the heart, aorta, and kidney were frozen immediately in liquid nitrogen (LN_2) and stored at −80°C until

mRNA analysis. Aorta were stripped of adventitial fat during immersion in phosphate-buffered saline (PBS) solution and then collected in RNAlater® solution (Ambion, Austin, TX), stored overnight at 4°C and transferred to a vial for storage at −80°C. Further details of the specimen preparation and storage phase of the study are provided elsewhere (48).

RESULTS AND DISCUSSION

We compared the effects of an MR antagonist and aldosterone synthase inhibitor (FAD286) in uninephrectomized Sprague-Dawley rats treated with either high salt intake and Ang II (1 μg/hr) or vehicle for 8 weeks. FAD286 significantly decreased plasma aldosterone after Ang II/salt treatment (139 ± 10 pg/mL in Ang II/salt + FAD26-treated rats vs. 472 ± 59 pg/mL in rats treated with Ang II/salt alone at 4 weeks; $P < 0.05$), whereas spironolactone increased aldosterone in Ang II/salt-treated rats (not shown). Spironolactone and FAD286 prevented the hypertensive response to uninephrectomy/salt alone but not to uninephrectomy/salt + Ang II. Both spironolactone and FAD286 attenuated Ang II/salt-induced cardiac and aortic medial hypertrophy and cardiac interstitial fibrosis, and neither drug decreased cardiac perivascular fibrosis (Figure 1).

Ang II/salt caused albuminuria, azotemia, renal and renovascular hypertrophy, glomerular injury (Figure 2), and tubulointerstitial fibrosis. Spironolactone and FAD286 equivalently prevented these effects.

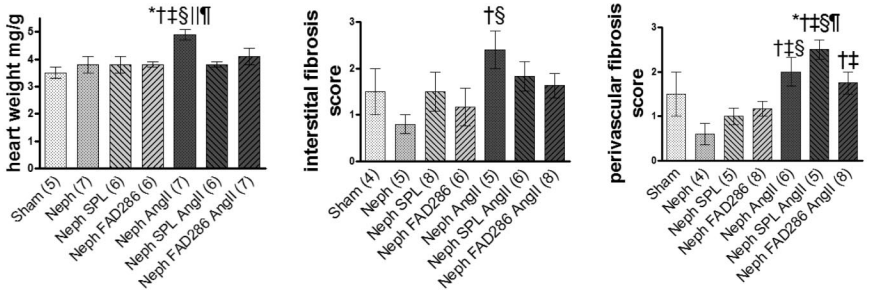

FIG. 1. Effect of the MR antagonist spironolactone (SPL) or the aldosterone synthase inhibitor FAD286 on heart weight (*left panel*), cardiac interstitial fibrosis (*center panel*), and perivascular adventitial fibrosis (*right panel*) in rats treated with uninephrectomy (NEPH) and 8-weeks of angiotensin (Ang II) infusion. For *post hoc* comparison, $^*P < 0.01$ versus sham, $\dagger P < 0.05$ versus uninephrectomy; $\ddagger P < 0.05$ versus uninephrectomy+spironolactone; $\S P < 0.05$ versus uninephrectomy+FAD286; $\| P < 0.05$ versus uninephrectomy+Ang II+spironolactone; $\P P < 0.05$ versus uninephrectomy+Ang II+FAD286. Reprinted with permission (47).

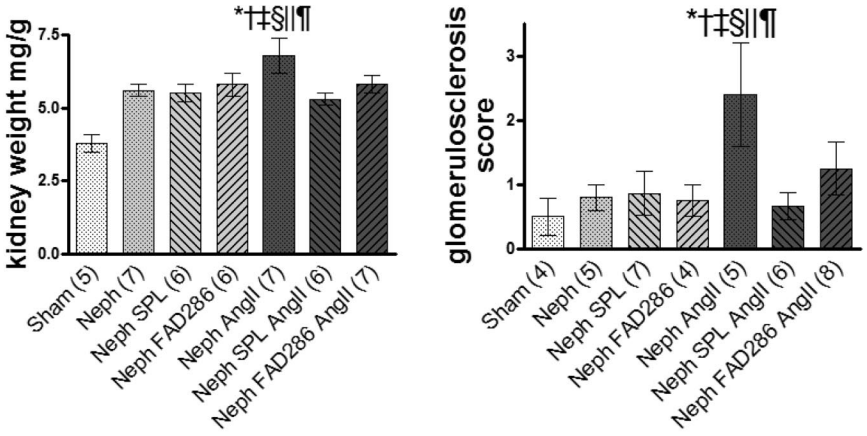

FIG. 2. Effect of the MR antagonist spironolactone (SPL) or the aldosterone synthase inhibitor FAD286 on kidney weight (*left panel*) and glomerular injury (*right panel*). For *post hoc* comparisons, $*P < 0.01$ versus sham; $\dagger P < 0.05$ versus uninephrectomy; $\ddagger P < 0.05$ versus uninephrectomy+spironolactone; $\S P < 0.05$ versus uninephrectomy+FAD286; $\| P < 0.05$ versus uninephrectomy+Ang II+spironolactone; $\P P < 0.05$ versus uninephrectomy+Ang II+FAD286. Reprinted with permission (47).

Effects on profibrotic gene expression varied. In the kidney, both spironolactone and FAD286 prevented Ang II/salt-induced PAI-1 and osteopontin expression. In the aorta, both drugs reduced osteopontin and TGF-β expression. In contrast, spironolactone enhanced Ang II/salt-stimulated PAI-1 expression in the aorta and perivascular areas of the heart, in accord with an MR-independent effect of aldosterone (47).

In short, low-dose MR antagonism and aldosterone synthase inhibition similarly decreased Ang II/salt-induced renal and cardiac hypertrophy and fibrosis, but the two procedures differed in their effect on profibrotic gene expression.

Contribution of endogenous aldosterone to inflammatory gene expression following acute Ang II infusion. To test the hypothesis that Ang II induces profibrotic gene expression through endogenous aldosterone, we measured the effect of a 4-hour infusion of (600 ng/kg/min) Ang II on tissue expression of mRNAs for PAI-1, preproendothelin-1 (ppET-1), TGF-β, and osteopontin expression in WT mice, AS$-/-$ mice, and AS$-/-$ mice treated with aldosterone (either 500 ng/day for 7 days or 250 ng as a concurrent 4-hour infusion). Ang II increased aldosterone levels in WT ($P < 0.001$) but not in AS$-/-$ ($P = 0.88$) (Figure 3). Aldosterone given for 7 days normalized basal aldosterone concentrations in AS$-/-$ mice; however, there was no further effect of Ang II on aldosterone ($P = 0.21$) (Figure 3). Giving exogenous

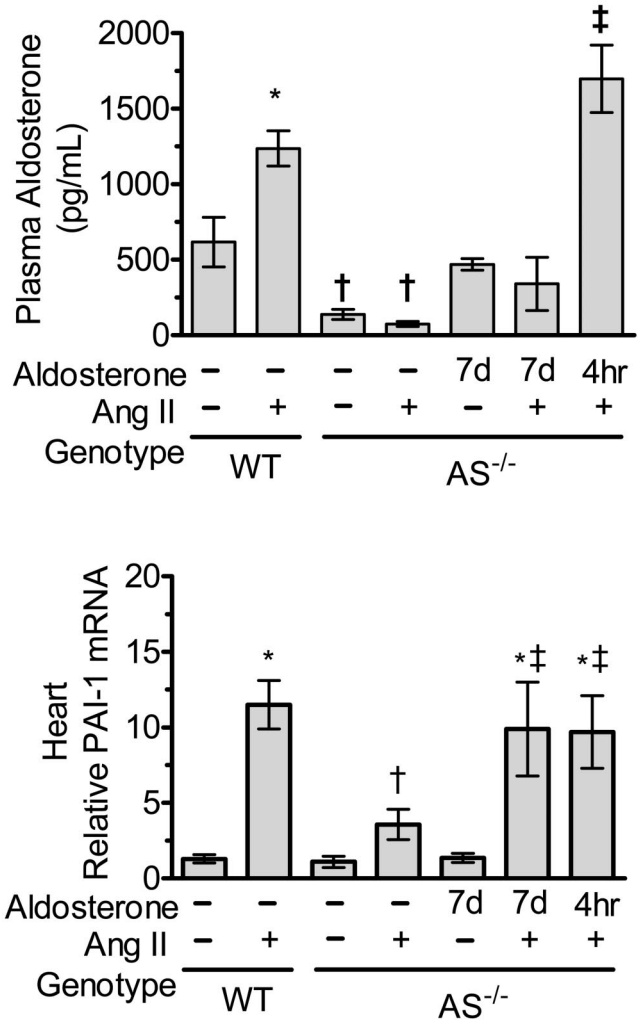

FIG. 3. *Top panel:* Effect of 4-hour angiotensin II (Ang II) infusion on circulating aldosterone concentrations in WT and AS−/− mice in the presence and absence of pretreatment (7 days) or concurrent treatment (4 hours) with aldosterone. *$P < 0.001$ versus WT-vehicle; †$P < 0.02$ versus either WT group; ‡$P < 0.01$ versus all groups. *Bottom panel:* Effect of 4-hour angiotensin II (Ang II) infusion on relative expression of mRNA for plasminogen activator inhibitor-1 (PAI-1) in the heart in WT and AS−/− mice in the presence and absence of pretreatment (7 days) or concurrent treatment (4 hours) with aldosterone. *$P < 0.05$ versus vehicle in the same genotype; †$P < 0.05$ versus WT-Ang II or AS−/−-aldosterone (7 days)-Ang II; ‡$P < 0.01$ versus AS−/−-Ang II. Reprinted with permission (48).

aldosterone for 4 hours to AS−/− mice successfully mimicked the effect of acute Ang II infusion on aldosterone concentration in WT mice.

Basal cardiac (Figure 3) and aortic PAI-1 and prepro-endothelin-1 (ppET-1, not shown) expression were similar in WT and AS−/− mice. Ang II-stimulated PAI-1 ($P < 0.001$) and ppET-1 expression ($P < 0.05$) were diminished in the hearts of AS−/− mice; pre-treatment with aldosterone for 4 hours or 7 days restored PAI-1 (Figure 3) and ppET-1 mRNA responsiveness to Ang II in the heart. In contrast, Ang II increased PAI-1 ($P < 0.05$) and ppET-1 expression in the aortas of AS−/− as well as those of WT mice, indicating that the effects of Ang II on aortic PAI-1 and ppET-1 expression were independent of aldosterone in the aorta (not shown). In the kidney, basal PAI-1, ppET-1, and TGF-β mRNA expression were increased in AS−/− as compared with WT mice, and correlated with plasma renin activity. Ang II did not stimulate osteopontin or TGF-β expression in the heart or kidney.

In summary, endogenous aldosterone contributes to the acute stimulatory effect of Ang II on PAI-1 and ppET-1 mRNA expression in the heart but not in the aorta. In the kidney, renin activity correlates with renal basal profibrotic gene expression. (This last finding is consistent with data collected by Nancy Noble's group showing that renin increases PAI-1 expression in the kidney via an AT_1-independent mechanism (49).)

On the basis of these findings, we have now completed a study comparing the effect of spironolactone and genetic aldosterone synthase deficiency, alone and together, on end-organ damage following chronic (8-week) treatment with Ang II and a high-salt diet. The study data suggest that MR antagonism and genetic aldosterone synthase deficiency have comparable effects on cardiac hypertrophy and fibrosis and on aortic remodeling, suggesting that endogenous aldosterone induces cardiovascular injury via the MR. The effects of MR antagonism and genetic aldosterone synthase deficiency on albuminuria and glomerular injury were additive, suggesting that activation of the MR by aldosterone, as well as by other ligands, contributes to glomerular injury.

Relevance to humans: Ang II induces inflammation in humans through an MR-dependent mechanism. What is the relevance of these findings in rodents to the human situation? We have previously reported that Ang II induces inflammation in humans via MR activation (50). First, we infused aldosterone or vehicle overnight in normal subjects in a double-blind crossover study (Figure 4). We found that aldosterone infusion increased circulating concentrations of the inflammatory cytokine interleukin-6 (IL-6), whereas vehicle did not. We further assessed the contribution of endogenous aldosterone

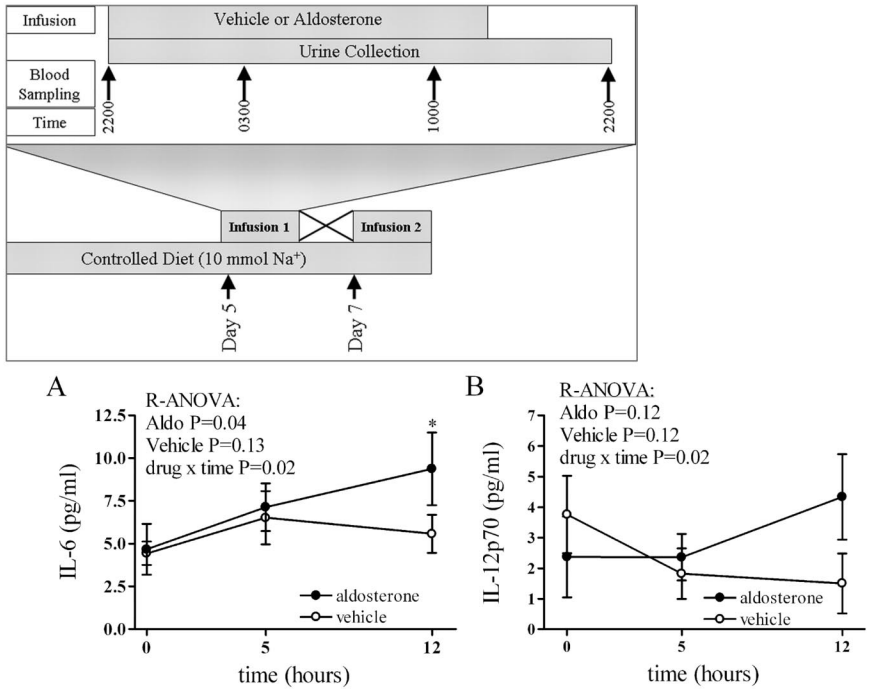

FIG. 4. Effect of overnight aldosterone infusion or vehicle on circulating interleukin-6 (IL-6) (*panel A*) or interleukin 12p70 (IL-12p70) (*panel B*) concentrations in normal healthy human subjects studied under low-salt conditions. *Top panel:* study protocol. Reprinted with permission (50).

and MR activation to the proinflammatory effects of Ang II in humans. To do this, we measured the effect of acute Ang II infusion on circulating IL-6 concentrations in a randomized, cross-over study in which subjects were pretreated with spironolactone or placebo (Figure 5). Norepinephrine was infused as a pressor control. We found that both Ang II and norepinephrine increased blood pressure and that spironolactone did not alter blood pressure. Ang II, but not norepinephrine, increased IL-6 concentrations, and pre-treatment with spironolactone blocked this effect. Lastly, we and other groups have reported that circulating inflammatory cytokines and PAI-1 concentrations are increased in humans during low-salt intake when the renin-angiotensin-aldosterone system is activated in comparison with their concentrations during a high salt intake (51, 52).

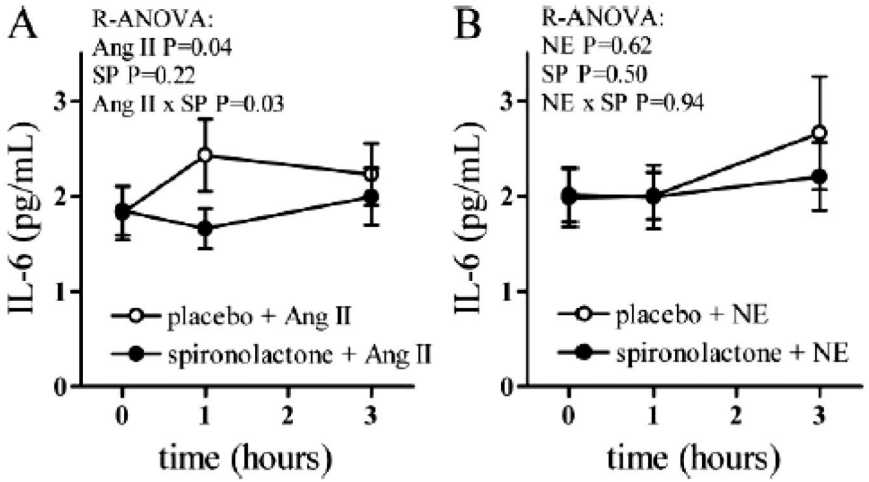

FIG. 5. Effect of 3-hour infusion of angiotensin II (Ang II) (*panel A*) or norepinephrine (NE) (*panel B*) on circulating IL-6 concentrations in normal healthy human subjects studied under high-salt conditions. Reprinted with permission (50).

SUMMARY

Aldosterone causes cardiac, vascular, and renal inflammation and fibrosis. PAI-1 contributes to the progression of aortic remodeling and renal disease, but not to cardiac fibrosis in the presence of aldosterone. Studies done with pharmacologic aldosterone synthase inhibitors and aldosterone synthase-deficient mice suggest that aldosterone is the primary ligand acting at the MR to cause cardiovascular and renal injury. Aldosterone also induces inflammation in humans through an MR-dependent mechanism.

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DISCUSSION

Gotto, New York: Beautiful presentation. When I was being interviewed by the Peter Bent Brigham Hospital for internship, George Thorne asked me to explain why patients with primary hyperaldosteronism didn't get edema. Well this, association that you're talking about with obesity, would that be secondary or primary?

Brown, Nashville: This is a debated subject. We know that aldosterone is produced in adipocytes, for example, so that is non-adrenal production. Ted Goodfriend has shown that there are fatty acids that increase aldosterone production by glomerulosa cells; however, as I said, some of the data that we are uncovering suggest that the converse may be true: that aldosterone itself may be contributing to obesity, or at least to the evolution from metabolic syndrome to diabetes.

Billings, Baton Rouge: In the first talk that we heard, Ron Hoffman spoke about primary myelofibrosis and inflammation with massive splenomegaly and hepatomegaly,

and I'm wondering whether if plasminogen activator inhibitor-1—PAI-1—has been looked at in primary myelofibrosis, and whether or not there is any relationship to that type of fibrotic change.

Brown, Nashville: That is a very good question, but I think that Doug Vaughn ought to answer that question. He will answer it later, and is going to show data, I am sure.

Abboud, Iowa City: Great talk, Nancy. I wasn't sure about the effect of low salt. Does that increase the inflammatory response, as you said, or decrease it?

Brown, Nashville: Low salt intake, by virtue of increasing aldosterone, increases the inflammatory response. What you may be getting at is that, in infused aldosterone models, there is a requirement for high salt in order to see cardiac fibrosis. A high salt intake would clearly suppress PAI-1, and I said that PAI-1 is protective in the heart, so there may be something there. But the relationship between high salt intake and inflammation is one that we really don't understand yet.

Abboud, Iowa City: The recommendation for a low salt intake in hypertension has to do with the increasing recognition of the inflammatory response as being very important in terms of the vascular damage in hypertension.

Brown, Nashville: Yes. Well, remember that these low salt diets that were giving are not something that you ever have to worry about patients ingesting on their own.